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Gene transfer with lipospermines and polyethylenimines

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Abstract

It is an obvious and basic principle that to be efficient, gene therapy requires effective gene transfer followed by adequate gene expression. However, getting DNA, a pro-drug, into the cell and into the nucleus, remains a crucially limiting factor. Even recombinant viral methods still show poor performances in clinical situations and non-viral methods are considered classically to be of yet lower efficiency. Here, we consider the mode of action, the nature of the complexes formed with DNA and the transfection potentials of two categories of inert, cationic vectors, the lipospermines and polyethylenimine. Both are among the best vectors currently available for in vitro work. Moreover, polyethylenimine is proving to be a versatile and effective carrier for different in vivo situations, especially for delivering genes into the mammalian brain.
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Keywords: Cationic lipids: Transfectam; Exgen 500; In vivo gene transfer; In vitro gene delivery; Mammalian brain: Cationic polymers

Contents

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1. Introduction

1.1. Gene therapy's bottle neck is gene transfer

By definition, gene therapy requires effective gene transfer followed by adequate gene expression. However, currently available gene transfer techniques are failing to provide sufficient expression of the desired protein product. Indeed, DNA is but a pro-drug, its transfer into the cell must be followed by a cascade of events beginning in the nucleus and culminating in the synthesis of a large number of effector protein molecules. Vector systems must thus deliver the exogenous DNA to the nucleus. Many recombinant viral vectors have been engineered to carry out this task, as during evolution, viruses have developed sophisticated cell break-in methods that can theoretically be exploited. These methods include cell targeting, efficient cell membrane rupture mechaniams and nuclear transport. Membrane rupture can occur either directly at the cell surface or occur after endocytosis. In each case, the viral fusogenic protein undergoes a major conformational change induced either by binding to a cell surface receptor or by the acidic nature of the endosomal compartment. Chemists examining such complex molecular sequences may well be daunted. Yet, synthetic non-viral gene transfer systems, however basic, will be of great potential to the gene therapy field just as soon as they show appropriately adequate in vivo transfection capabilities. However, as emphasised by the attempts to apply gene therapy to cystic fibrosis or melanoma patients, we know that this criterion is far from being satisfied.

2. Lipospermines and polyethylenimines — concepts and structural features

Chemical design is not constrained by the need for replication that is characteristic of a biological system and can therefore explore and exploit a larger spectrum of candidate molecules for a given task. With some imaginative leads and a great deal of 'evolutionary' trial and error, two classes of synthetic vectors have been developed over the last decade. These compounds, whether lipids or polymers, are all cationic, like their classical predecessors used for gene transfer in vitro (calcium phosphate, DEAE–dextran). On complexing with DNA, they cause several plasmid molecules to condense into sub-micrometric particles.

Lipospermines [1] differ mostly from the other lipids used in gene transfer by their headgroup (Fig. 1, see Transfectam and DPPES). Spermine is a natural polyamine, which can be found, for example, in the eucaryotic cell nucleus. Its high charge density potential allows it to condense DNA into small toroïdal structures [2]. When spermine is coupled to a hydrophobic tail, the resulting lipid has a bulky headgroup, leading to a conical shape with a high radius of curvature. In contrast, monovalent, cationic lipids have a small headgroup, leading to a more or less cylindrical molecule. When the lipids are dispersed in an aqueous phase, the size and structure of the resulting multi-molecular assembly will be driven by their shape. In fact, lipospermines form small micelles, whereas monovalent, cationic lipids generally form bilayers. This difference is also observed upon mixing with DNA. Electron microscopy of Transfectam–DNA complexes formed at a 6:1 charge ratio shows well-defined structures (50 to 100 nm). These structures are found either alone or aggregated in larger complexes (100 to 400 nm). Within a single complex, one could either see parallel lines or circles. This may indicate that Transfectam forms tubular micelles, with the DNA being wrapped around and between them (Fig. 2A).

When these complexes are used in transfection,

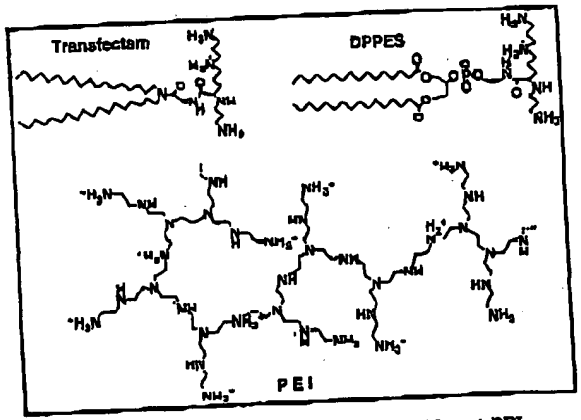

Fig. 1. Structures of Transfectam, DPPES and PEI.

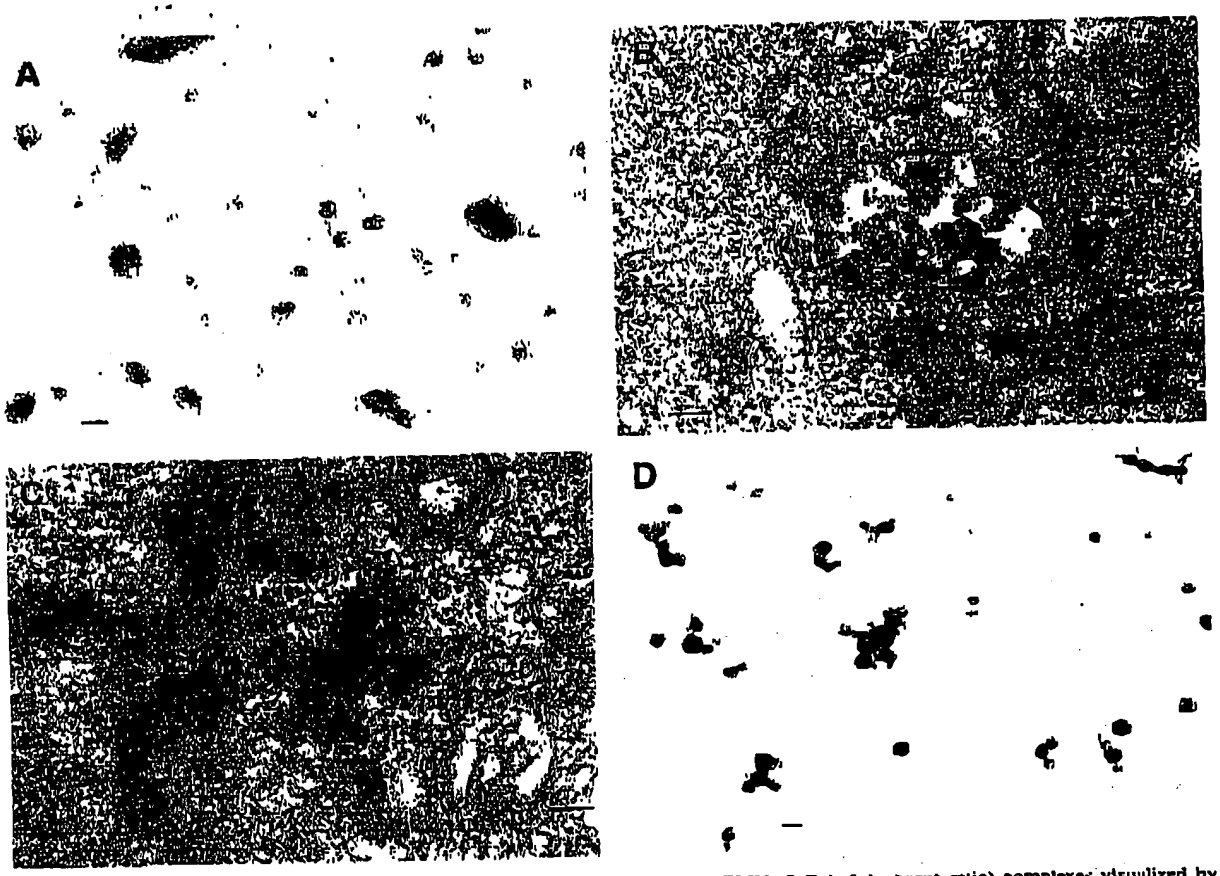

Fig. 2. Electron micrographs of (A) Transfectam–DNA (10 μg of DNA/ml of pCMV–β-Gal; 6:1 charge ratio) complexes visualized by negative staining; (B–C) Transfection of 3T3 cells with Transfectam–gold-labeled DNA (2 μg of DNA/10^5 cells; 6:1 charge ratio) complexes endocytosed (B) or free in the cytoplasm (C). (D) PEI–DNA (10 μg of DNA/ml; 10:1 nitrogen-to-phosphate ratio) complexes visualized by negative staining. Bar is 100 nm.

they stick to the cell surface within minutes and are internalized [3]. Cells transfected with Transfectam complexed to gold-labeled DNA show that endocytosed complexes contain both the lipid and the nucleic acid (Fig. 2B). In some cases, they are found free in the cytoplasm (Fig. 2C) and/or in the perinuclear region.

Cationic lipids give variable transfection efficiencies that depend both on the chemical structure of the vector and on the cell type targeted. Even so, whatever the cell type used, the lipopolyamines constitute one of the most efficient vector classes [4–9]. The polyamine headgroup must in itself carry multifunctional properties that are important for gene transfer efficiency, since performance cannot be improved either by the addition of fusogenic lipids or with nuclear localisation signals [10]. Moreover, when the potentiometric protonation states of the amines were measured, it was found that, at physiological pH, only three of the four nitrogens in the spermine head were cationic (Fig. 1). The pKa of the last amine is 5.5 [11], half-way between the extracellular and intralysosomal pH values, a clue to a possible buffering property that could well be exploited. In contrast, monovalent cationic lipids have a headgroup containing a quaternary ammonium,

which is constitutively charged, preventing them from buffering endosomes. The buffering hypothesis is bolstered by results obtained with other cationic polymers, the polyamidoamine dendrimers [12]. These quasispherical macromolecules bear a large number of amine groups and, again, as for the lipopolyamines, not all of these amines are protonated at physiological pH [13].

So, by observing two completely different cationic vectors, a lipid and a polymer, we came to the same question: Is there is a causal relationship between the overall buffering capacity of a vector under physiological conditions and its transfection possibilities? Accordingly, a number of macromolecular compounds bearing high amine group densities were considered for synthesis. Such cationic compounds would still be able to compact DNA, but, due to the repulsion predicted between like charges at close proximity, they would not be fully protonated at physiological pH. As it turned out, there was no need to start synthesising candidates, as the ideal molecule was already available. In the commercially available polymer polyethylenimine (PEI, Fig. 1), one in every three nitrogens is in an amine group and the overall protonation level increases from 20 to 45% between pH values of 7 and 5 [14]. Moreover, the compound was identified over 50 years ago and its innocuity has been demonstrated by its intensive and various uses, e.g., in water purification, ore extraction and in shampoos. In fact, PEI is the cationic polymer that has the highest charge density potential. Every third atom is an amino nitrogen that can be protonated. Commercially available PEIs form small complexes with DNA at high nitrogen-to-phosphate ratios (Fig. 2D). Their interaction with negatively charged plasma membranes is fast and, after only 2 h, numerous complexes can be found in endosomes (A.M. Stoffan, personal communication). Here again, complexes can be found free in the cytoplasm.

3. Results in cell lines and primary culture

3.1. Centrifugation and condensing DNA in small volumes improve efficiency, especially when serum is present during transfection

When a cationic lipid is used at an excess ratio of cationic charges-to-nucleic acid phosphates, the resulting nucleo–lipid particles fix to the cell surface. Indeed, in vitro, electrostatic interactions between the negatively charged cell membranes and the positively charged DNA–lipid complexes are enhanced by increasing the overall charge of the complexes, which is achieved by increasing the ratio of lipid to DNA. The transfection efficiency is increased concomitantly with the charge ratio, until toxicity appears. This coarse optimization can, however, be much more finely tuned. One approach was to consider that the transfection efficiency of small vector–DNA complexes may be limited by brownian motion, as recently described for retroviruses [15]. To counter this, we used centrifugation (5 min at 280 *g*) of cells covered by the transfection mixture, and found that it increased both PEI and Transfectam transfection efficiencies by up to 50-fold (Fig. 3; [16]).

The presence of serum generally drops the efficiency or increases the variability of many gene transfer vectors. This may hamper their use in vivo. Sequential addition of Transfectam over DNA has been shown to produce much more efficient complexes, especially in the presence of serum (Fig. 3C).

In vivo applications may also require a high concentration of complexes. Transfectam perfectly fulfils these requirements, since even a tenfold concentrated solution increases the efficiency compared to the 'regular' protocol (Fig. 3A, extreme left bar).

Finally, the best results, in the presence or absence of serum, are obtained when these different approaches for optimization are combined (Fig. 3B Fig. 3D, extreme right bar). Under conditions that can be considered as most relevant for in vivo conditions (blocks C and D), optimization improves transfection by three orders of magnitude (left bar to far right bar).

3.2. PEI is versatile and provides high levels of gene expression in vitro

We tested the transfection efficiency of this polymer, comparing it to that of lipopolyamines on a large variety of cell lines and primary cultures. The results are most promising, showing efficiencies that are at least as high as those achieved with the best currently available synthetic vectors ([17], Fig. 4),

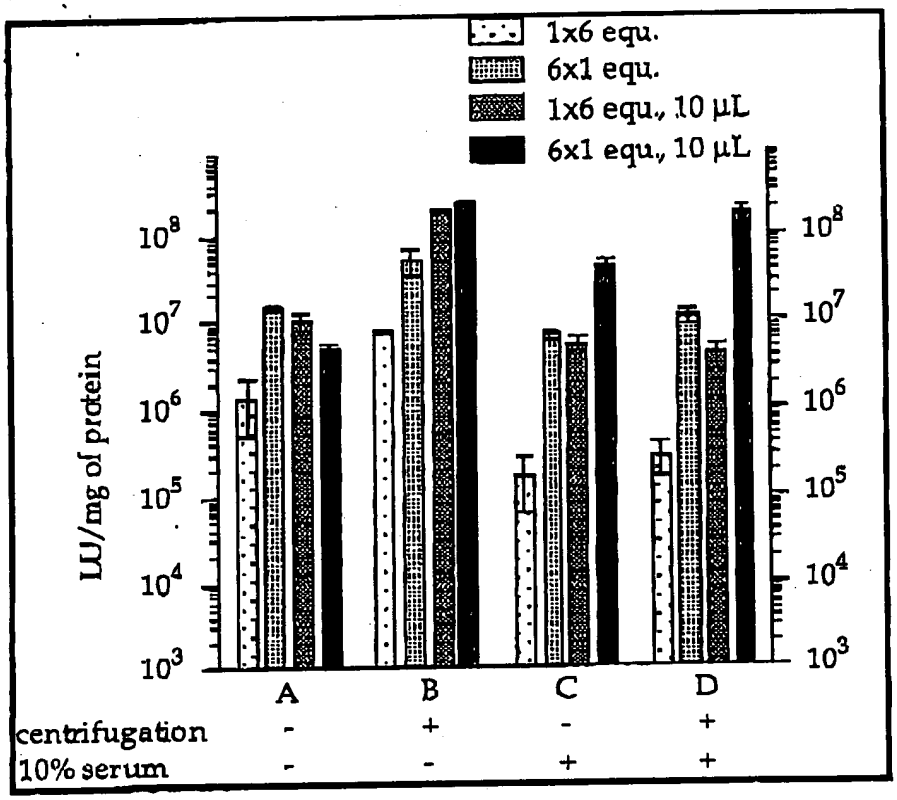

Fig. 3. Transfection can be improved by combining the sequential addition (6 × 1 charge equivalent) of lipid, by using high complex concentrations and by centrifuging the complexes onto the cells. Transfectam–DNA (2 µg of pCMV–Luc/10^5 3T3 cells; 6:1 charge ratio) were prepared using either the regular protocol (1 × 6 charge equivalents) or the multi-step procedure in 100 µl of 0.15 M NaCl (unless specified) and poured over the cells (in DMEM, with or without 10% serum). In experiments B and D, the cells were then centrifuged (5 min, 280 *g*). After 2 h, serum was added (if required) and the transfection activity was monitored after 24 h. Results are expressed as light units per mg of protein and mean±SE are given ($n = 3$).

indicating an entirely new function for this simple molecule.

We also compared PEIs of various molecular weights, both branched and linear. PEIs modified with epichlorhydrine or ethoxylated, polypropylenimines of different sizes, 57 kDa polyallylamine, 32 kDa polyhistidine, chitosan and $(3,10)_n$-polyamine to polyamidoamine dendrimer and 50 kDa polylysine on 3T3 cells, using the firefly luciferase reporter gene (Fig. 5).

We showed that only PEIs with molecular weights above 2 kDa and dendrimers are very efficient in gene transfer. Yet, PEI is a much simpler and cheaper molecule. Moreover, PEI can be chemically modified (i.e. ethoxylated or modified by epichlorhydrine) without loss of activity. This result shows that they can be an ideal base for chemical modifications (see Section 5).

4. In vivo gene transfer mediated by Transfectam and polyethylenimines

4.1. Low charge ratios and the addition of dioleoylphosphatidyl ethanolamine (DOPE) can be used for in vivo Transfectam-mediated gene transfer

In some ways, the conditions described in the above experiments are mimicking those found in

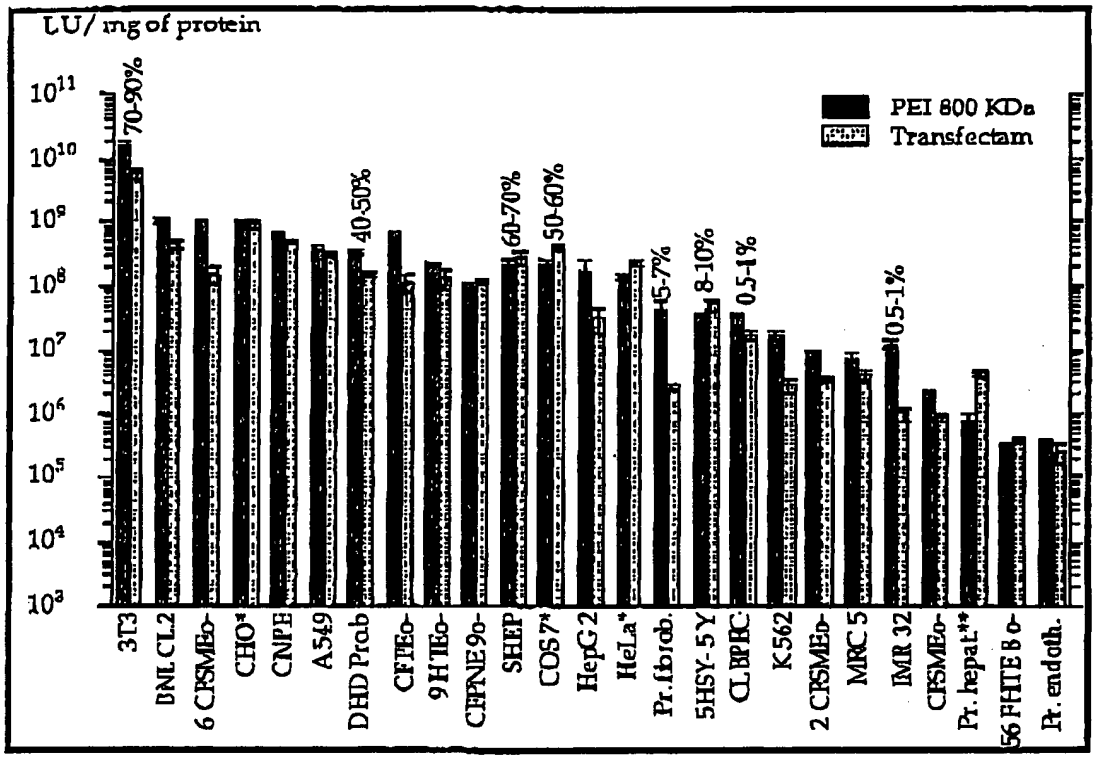

Fig. 4. Comparative transfection of various cell types with Transfectam and PEI. Cells were transfected with 2 μg of pCMV–Luc and Transfectam (6 equiv.) according to the stepwise addition protocol (except for *, where they were added simultaneously) or PEI 800 kDa (9 equiv.). Plates were centrifuged for 5 min at 280 g (**, hepatocytes were centrifuged for only 5 min at 44 g). Percentages indicated over the bars correspond to the number of blue cells counted after transfection with 2 μg of pCMV–β-Gal plus PEI.

vivo, in particular, by the use of high concentrations of complexes and even the use of centrifugation. Indeed, this latter step is relevant in that it plays out the circumstances of topical injection into a tissue, where DNA–lipid particles are soon in close contact with the surrounding extracellular matrix and cells. However, the use of charged particles has proven inefficient for gene transfer in a number of in vivo models. This is particularly marked for cationic polymers such as poly-L-lysine. With regard to lipopolyamines, such as Transfectam and Lipofectin, which have been on the market for eight years or so, only recently have reports appeared showing their potential use for certain in vivo situations. Transfectam can provide reasonable levels of transfection when used alone, without the adjunction of a neutral lipid, in the chick embryo [18] and in the mouse embryo transfected through the introduction of DNA complexed with lipid into the maternal blood supply [19].

We have also succeeded in defining conditions that provide high levels of in vivo transfection in the brains of newborn mice using Transfectam [20]. As shown in Fig. 6, we found that, in this model, the best levels of transfection were obtained when using a low ratio of positive charges (supplied by Transfectam) to negative charges (carried by the DNA). Moreover, the addition of a two molar excess of the neutral lipid DOPE significantly enhanced transfection (Fig. 6).

Other authors have also reported that mixtures of cationic lipids and DOPE, used at low overall charge

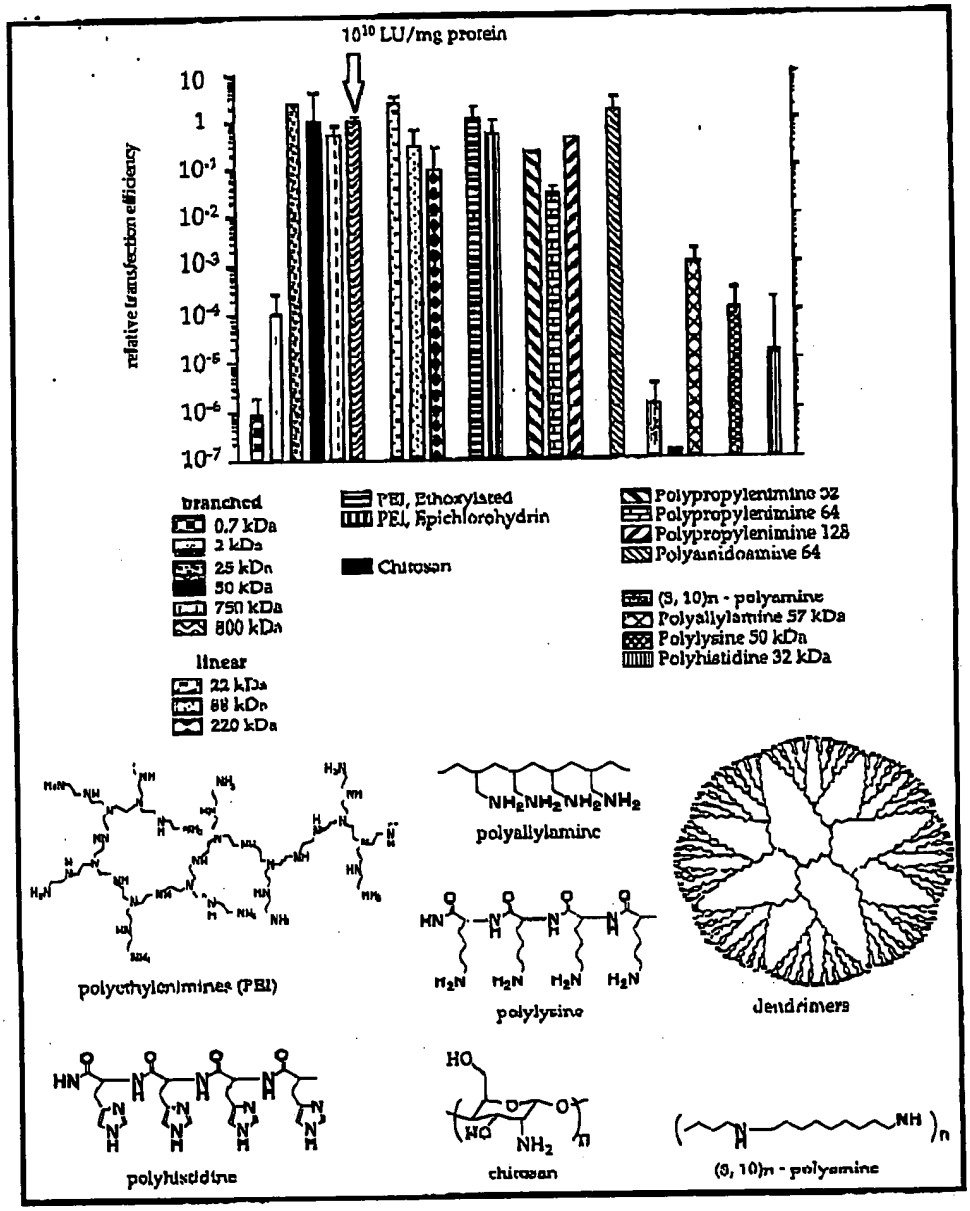

Fig. 5. Relative in vitro transfection efficiencies of various cationic polymers. 3T3 cells were transfected with 2 μg of pCMV–Luc complexed to polymers at their optimal charge ratio. Immediately after the addition of complexes to the cells, plated in 24-multiwell dishes, the plates were centrifuged at 280 *g* for 5 min. After 2 h, foetal calf serum (10%, v/v) was added and luciferase expression was monitored after 24 h. Transfection efficiencies are expressed as light units (LU) relative to PEI 800 kDa (10^{10} LU/mg of proteins), and means±SEM are given ($n = 3$). Chitosan (Aldrich, Saint Quentin Fallavier, France) has an average molecular weight of 1000 kDa and is fully deacetylated. Dendrimer sizes are expressed as the number of terminal primary amines. PAMAM and polyethylenimine dendrimers were kind gifts from Prof. Frank Szoka (University of California, San Francisco, CA, USA) and Prof. Bert Meijer (Eindhoven University of Technology, Netherlands), respectively.

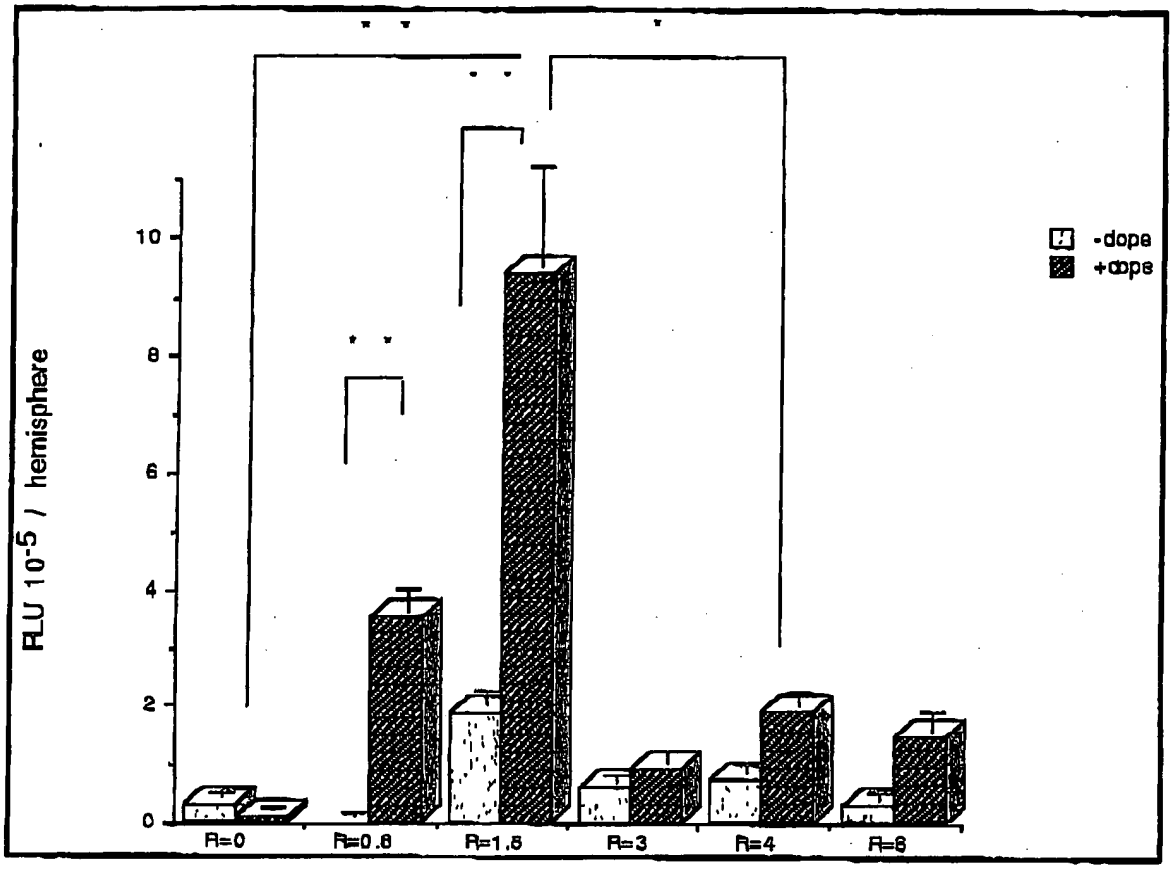

Fig. 6. Mixing Transfectam and DOPE in a 1:2 molar ratio provides efficient gene transfer in the newborn mouse brain. Effects of the charge ratio (R = lipid cation–DNA anion) and of the addition of a twofold molar excess of the neutral lipid, DOPE, on the efficiency of Transfectam-mediated gene transfer. Newborn mice received intrastriatal injections containing 2 μg of pCMV–Luc in 2 μl of 5% glucose. Mice were sacrificed 24 h later and luciferase activity was measured in brain homogenates. DNA was applied alone or compacted with Transfectam (grey columns) or a 1/2 molar ratio of Transfectam–DOPE (striped columns). Values are means±SEM, $n = 10$ throughout. $^{**}P \leq 0.001$; $^{*}P \leq 0.05$.

ratios, can deliver genes in vivo, either with Lipofectin (which is commercialised as a mixture of a cationic lipid and DOPE, see, for example, ref. [21]) or with Transfectam [22,23]. It is worth noting that in the particular case of the adult brain, the only successful uses of cationic lipids have been those employing very low amounts of lipid (usually Lipofectin), but the amounts used are insufficient to fully condense the DNA injected. Thus, it is possible that the low levels of transfection observed under these conditions are due to the activity of naked or free DNA escaping from interactions with the low amounts of lipid. This hypothesis is supported by the fact that we have recently shown that free DNA can provide low, but reproducible, levels of transgene expression in the mature mammalian brain [24].

4.2. In vivo veritas: PEI can be used in different in vivo situations

One of the most promising aspects of PEI-based gene transfer comes from results obtained in vivo

which show PEI to have the edge on the other vectors. Indeed, the main limitation of current non-viral gene transfer methods is their relatively low efficiencies in vivo, with cationic lipids often requiring dilution with neutral or amphiphilic lipids to achieve delivery (see above). No such molecular juggling with neutral compounds is required with PEI. In both the adult and the newborn mouse brain, PEI–DNA complexes provide levels of transfection that are equal to those found in vitro for the same amount of DNA applied to primary neuronal cultures (up to 10^6 RLU per μg of DNA injected, Fig. 7). As shown in Fig. 7, the best levels of expression in both models are obtained with polymers that have a mean MW of 25 kDa (commercialised by Aldrich). In the adult brain, we have used double immunostaining with antibodies against cell-specific markers and transgene products to show that both neurons and glia can be transduced by PEI transfection in vivo. Moreover, toxicity is low, no mortality being observed in injected animals and no necrosis at the site of injection [25]. Also of interest is that when transfecting neuronal cells in culture, no interference with membrane excitability is seen [26].

In both models, i.e., newborn and adult central nervous systems, complexes with low overall charges provide the best transfection efficiencies. Ratios of six or nine amines per DNA phosphate were found to be optimal in the adult and immature brain, respectively. Theoretically, these ratios produce complexes bearing net charges around neutrality, as only one in five of the protonatable amines carried by the PEI are in fact protonated at pH 7. Such complexes would be expected to diffuse widely in the extracellular matrix (whereas highly charged cationic complexes, such as those used in the in vitro situations, would probably stick to substrates around the injection site). We do in fact find high diffusibility of complexes in the newborn brain. As shown in Fig. 8, following injection of 2 μl of complexes into the striatum, expression is found in the olfactory bulb, which is over 5 mm distant from the injection site. Similar diffusion can even be found in the adult brain [25], a much more dense and compact tissue than the immature nervous system.

Thus, as these experiments in the mouse show, PEI appears to be an ideal vector for in vivo gene transfer into the mammalian brain at different stages

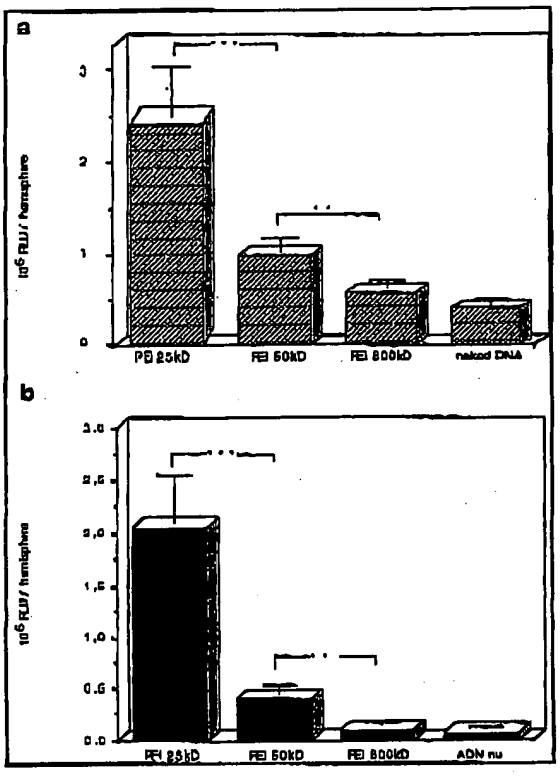

Fig. 7. Efficiency of gene transfer is inversely related to mean PEI polymer size. (A) Comparison of transfection efficiencies obtained with 2 μl of 0.5 μg/μl CMV–Luc in 5% glucose either alone (naked DNA) or complexed with 25, 50 or 800 kDa PEI. All PEIs were used at a ratio of nine amines per DNA phosphate. Complexes were injected into the striata of anesthetized newborn mice. Expression was measured 24 h later. (B) Comparison of transfection efficiencies obtained with 5 μl of 0.5 μg/μl CMV–Luc in 5% glucose either alone (naked DNA) or complexed with 25, 50 or 800 kDa PEI. All PEIs were used at a ratio of six amines per DNA phosphate. Complexes were stereotaxically injected into the cortex of anesthetized adult mice. Expression was measured 72 h later.

of development. Future studies will determine if the methodology can be adapted to other tissues.

5. Conclusion and perspectives

The sequence of events that we hypothesize to account for the remarkable transfection properties of

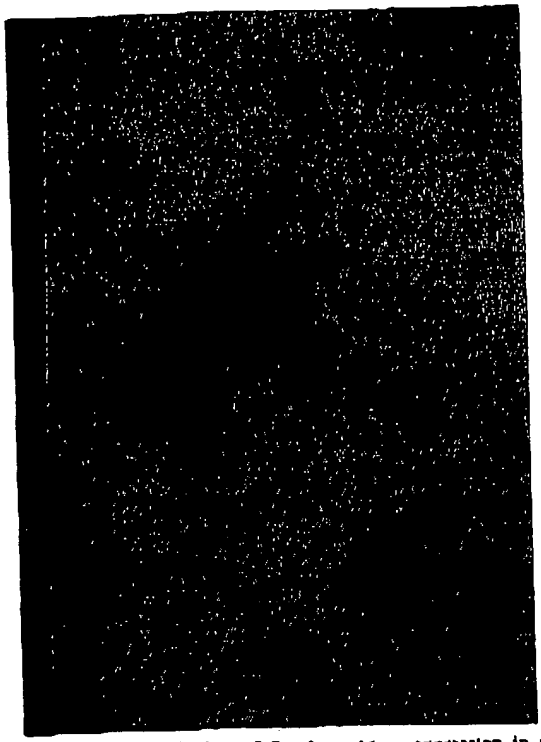

Fig. 8. Spatial distribution of β-galactosidase expression in the newborn mouse brain after transfection of 1 μg DNA (CMV–β-Gal, Clonetech) complexed with PEI (volume 2 μl) into the striatum. A low power overall view of the clarified, delipidated brain is shown.

PEI are summarized in Fig. 9. The polycation–DNA complexes probably enter the cell by spontaneous endocytosis. During intracellular trafficking, the buffering capacity of the PEI will not only tend to inhibit the action of the lysosomal nucleases that have an acid optimal pH, but will also alter the osmolarity of the vesicle. The accumulation of protons brought in by the endosomal ATPase is coupled to an influx of chloride anions [27]. In the presence of PEI, there will be a net increase in the ionic concentration within the endosome, resulting in a swelling of the polymer by internal charge repulsion and osmotic swelling of the endosome, due to water entry. With the two phenomena occuring simultaneously, it is likely that endosomal life expectancy is sorely reduced! Taking into account the protonation level of PEI, we can expect that about a third of the nitrogen atoms in the molecule participate in the swelling action, making the molecule a virtual proton sponge. For gene therapy, the interesting aspect of this mechanism (which is somewhat 'primitive' compared to the mechanisms developed by viruses) is that it will lead to enhanced gene transfer, as the DNA introduced with the PEI will be rapidly liberated from the damaging endosomal environment. Thus, this molecule constitutes, per se, a promising vector for gene therapy and an ideal structural base for constructing more sophisticated vectors that could include supplementary functions such as cell-specific targeting ligands.

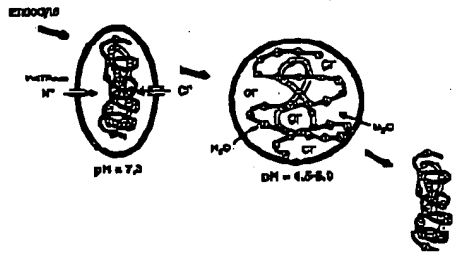

Fig. 9. The proton sponge hypothesis.

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